DAIDS

VIROLOGY MANUAL

FOR HIV LABORATORIES

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Compiled by

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and

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QUALITATIVE PBMC MICROCOCULTURE ASSAY

I. PRINCIPLE

Human immunodeficiency virus (HIV) has been shown to be the etiologic agent of Acquired Immunodeficiency Syndrome (AIDS). Isolation of virus specimens from AIDS patients and the presence of antibodies in their serum have helped to determine this etiologic association.

The qualitative macrococulture assay is one of the more sensitive virologic methods available for determining or verifying infectivity of peripheral blood mononuclear cells (PBMC). This procedure is usually performed in 25 cm^2 sterile flasks by coculturing 10^7 patient PBMC with an equal number of PHA-stimulated PBMC from a normal donor at a combined final concentration of 2 x 10^6 PBMC/mL. The coculture supernatant is tested twice weekly for HIV p24 antigen production which is used as a marker for viral replication. Fresh PHA-stimulated normal donor cells are added to the coculture once a week, and the culture is terminated at day 21 unless a positive result is confirmed at an earlier time interval.

In the event that an insufficient number of patients cells are available to perform a macrococulture, a qualitative micrococulture may be performed as an alternative method. The main differences in the methods are as follows:

Requires 2 x 10^6 patient PBMC's (1 x 10^6 assayed in duplicate);
Cocultured with 1 x 10^6 PHA-stimulated normal donor PBMC's;
Performed in a 24-well tissue culture plate;
5 x 10^5 fresh PHA-stimulated donor PBMC's in fresh media are added on day 7 and 14;
HIV p24 antigen is assayed on day 14 and on 21 if day 14 is negative; and
A positive culture well is defined by HIV p24 antigen levels of ≥30 pg/mL.

II. SPECIMEN REQUIREMENTS

Specimens should be stored at room temperature until processed. The assay utilizes heparinized, ACD or CPD anticoagulated peripheral blood from which PBMC are isolated by a Lymphocyte Separation Media gradient (see Methodology). Blood must be processed within 30 hours of collection. Patient PBMC s that are not used in the assay should be stored according to requirements as specified by each protocol (e.g., viable PBMC, dry pellet, etc.)

III. REAGENTS

Sterile PBS or HBSS, or Sterile 10x Dulbecco's PBS without Ca^{2+} or Mg^{2+}
Sterile water
Lymphocyte separation media (LSM or Ficoll-Hypaque)
Fetal bovine serum (FBS, sterile)

Antibiotics (e.g., Gentamicin - 10 mg/mL)
RPMI 1640 w/o L-Glutamine
L-Glutamine 200 mM
Interleukin-2 (IL-2)

Fetal Bovine Serum (FBS, sterile)

a. Thaw the 500 mL bottle of FBS completely.

b. Inactivate the FBS by immersing the bottle up to FBS level in a 56°C water bath for 30 minutes.

c. Label the bottle with the day of inactivation, and store at -20°C. Keep for up to 18 months from inactivation, or until expiration date of FBS, whichever is sooner.

Sterile 1x Dulbecco's PBS without Ca^{++} or Mg^{++}

a. Place 450 mL of distilled water into a 500 mL Nalgene bottle.

b. Add 50 mL of 10x Dulbecco's PBS without Ca^{2+} or Mg^{2+} to the bottle and mix well by inversion.

c. Open the bottle slightly and sterilize using the liquid setting on the sterilizer or sterilize by filtration through 0.22 μm Millipore filter.

d. Allow the Dulbecco's PBS to cool and label with a 6 month expiration date, or expiration of the Dulbecco's PBS, whichever is sooner and preparer's initials. Test for sterility.

e. Alternate: purchase sterile 1x PBS or HBSS

Coculture RPMI Media with 5% IL-2

a. Add 138 mL of heat inactivated FBS to a 500 mL bottle of RPMI 1640 w/o L-Glutamine.

b. Mix well by inversion.

c. Add 34.5 mL of Interleukin-2 to the mixture.

d. Add antibiotics to the mixture (e.g., 3.5 mL of Gentamicin).

e. Mix well by inversion.

f. Add 14.0 mL of 200 mM L-Glutamine to the mixture.

g. Label the bottle with the date of preparation, a 1 month expiration date, preparer's initials, and perform a sterility culture prior to use.

IV. EQUIPMENT AND SUPPLIES

Laminar flow hood (biosafety cabinet class II)
Gloves
Lab coat
Nalgene bottle, 500 mL
Sterile 50 mL conical graduated polypropylene centrifuge tubes
Sterile 15 mL conical graduated polystyrene centrifuge tubes
Sterile 75 x 100 mm tube
Serological pipettes, 10 and 2 mL
Polypropylene transfer pipettes
Disposable polystyrene blood dilution vials
Isoton II (Coulter)
Isoterge II (Coulter)
Bleach
Millipore filter, 0.22 μm
Tissue culture plate, 24-well
Pipette aid filler/controller (e.g., Drummond)
Tabletop centrifuge
Automated cell counter (e.g., Coulter Cell Counter)
Light microscope with 10x ocular
Cell dilutor (e.g., Dade dilutor)
Tissue culture incubator - 5% CO_2, 37°C and 98% humidity
56°C water bath

V. PROCEDURE

A. PATIENT SPECIMEN PREPARATION

No special patient preparation is required. Collect peripheral blood in heparin, ACD, or CPD vacutainer tube. If the patient has an abnormally low absolute lymphocyte count, the volume of blood will need to be adjusted. Do not refrigerate blood after collection. Deliver immediately to the laboratory and keep at room temperature prior to cell isolation. Isolate cells without delay (maximum, within 30 hours after collection). Log all specimens into the computer and obtain a specimen tracking number. Perform all procedures under a biological safety hood.

1. Mix the patient vacutainer tube(s) well by inverting several times.

2. Pour the whole blood into a 50 or 15 mL sterile conical centrifuge tube labeled with colored tape and the specimen log number.

3. Centrifuge at 400 x g for 10 min. at 24°C.

4. Remove the plasma which should be aliquoted into labeled tubes and stored frozen per specific protocol instructions.

5. Dilute the cells 1:2 by adding an equal volume of sterile Dulbecco's PBS.

6. Cap tubes and invert to mix several times.

7. Under or over layer the diluted blood with an equal to 2x volume of lymphocyte separation media.

8. Centrifuge at 400 x g for 30 min. at 24°C in the tabletop centrifuge.

9. Aspirate off the top layer of Dulbecco's PBS till approximately 0.5 cm above the PBMC layer.

10. Remove the PBMC layer with a sterile transfer pipette and place it into a 15 mL conical centrifuge tube.

11. Add Dulbecco's PBS in a 3:1 ratio to the cells.

12. Mix well by inversion.

13. Centrifuge the mixture for 10 min. at 400 x g at 24°C in the tabletop centrifuge.

14. Aspirate off the supernatant fraction and discard.

15. Add 10 mL of Dulbecco's PBS to the cells.

16. Resuspend the cell pellet by vigorously tapping the side of each tube with hand or by using a pipette.

17. Centrifuge the mixture for 10 min. at 400 x g at 24°C in the tabletop centrifuge.

18. Aspirate off the supernatant fraction and discard.

19. Add 1 mL coculture medium to the cell pellet.

20. Resuspend the cell pellet by vigorously tapping the side of each tube with hand or by using a pipette.

21. Remove 0.1 mL of the cell suspension into a 75 x 100 mm tube for a WBC count with an automated cell counter.

B. COCULTURE PROCEDURE

1. In two wells of a 24-well tissue culture plate, add 1 x 10^6 PHA-stimulated donor cells (see "Preparation of PHA-Stimulated Uninfected Donor Peripheral Blood Mononuclear Cells") and 1 x 10^6 patient PBMC's. Adjust final volume to 2 mL with coculture medium.

2. Put 2 mL of sterile water in corner wells to help maintain humidity.

3. Incubate at 37°C with 5% CO_2 .

4. At day 7 remove 1.0 mL of medium without disturbing cells. Replace with 1 mL fresh coculture medium containing 5 x 10^5 PHA-stimulated donor cells.

5. At day 14 remove 1.0 mL of medium without disturbing cells. Replace with 1 mL fresh coculture medium containing 5 x 10^5 PHA-stimulated donor cells.

6. Save day 14 supernatant fractions from duplicate wells separately and store at -30°C until analyzed for HIV p24 antigen by EIA.

7. Assay day 14 supernatant fractions for HIV p24 antigen and if both wells are positive, terminate culture; otherwise, continue culture until day 21.

8. Terminate culture on day 21, save supernatant fractions from duplicate wells separately, and store at -30°C until analyzed for HIV p24 antigen by EIA.

9. Assay day 21 supernatant fractions for HIV p24 antigen.

VI. RESULTS / INTERPRETATION

A. COCULTURE WELL RESULT: CRITERIA

The criteria for a positive or negative coculture well is based on VQA standardized HIV p24 antigen results obtained from the day 14 or day 21 culture samples. A culture well is considered positive if the HIV p24 antigen level is ≥30 pg/mL. A culture well is considered negative if the HIV p24 antigen level is <30pg/mL at both day 14 and day 21.

B. CULTURE RESULT: CRITERIA

The criteria for determining a positive, negative or indeterminate result for a qualitative micrococulture depends upon the HIV status of the patient.

1. For confirmed HIV-positive patients.

   If either well (or both wells) of a qualitative micrococulture is positive, the culture is considered positive. The culture is considered negative if both wells are negative.

2. For HIV-negative or HIV-undetermined patients.

   The culture is considered negative if both wells are negative and positive if both well are positive. If either well of a qualitative micrococulture is positive and the other negative, the culture is considered indeterminate and a subsequent specimen should be requested for testing.

VII. PROCEDURE NOTES

1. To reduce the chances of cross contamination and/or specimen mix-up, cells from only one patients should be set-up per 24-well tissue culture plate.

2. Laboratories performing this assay for ACTG or other DAIDS sponsored protocols, should be participating in and certified by the Virology Quality Assurance Quantitative Micrococulture certification program.

3. Cultures contain large quantities of HIV and are potentially infective to the technician handling the cultures. Gowns are required when working with any potential HIV containing specimens (i.e., peripheral blood, CSF, tissue specimens, etc.) and are changed weekly (daily if work being done in BSL-3 facility). Gloves must be worn whenever working with any potentially HIV containing specimens. Gloves should be changed often, especially if punctured or contaminated. All work must be performed in a certified biological safety laminar flow hood. All work areas in the laboratory must be wiped down with 10% sodium hypochlorite (bleach) at the beginning and end of the working day. The laminar flow hood must also be decontaminated with 10 sodium hypochlorite daily.

4. Blood collected in EDTA or CPD-A must always be diluted in saline or calcium and magnesium-free Dulbecco's PBS.

5. Lymphocyte separation media, coculture medium, and diluent used (Dulbecco's PBS or saline) must be at room temperature to prevent clumping of cells.
6. Inspect all 15 mL polystyrene and 50 mL polypropylene conical centrifuge tubes for cracks prior to use. Loss of cell suspensions will occur when cracked tubes are centrifuged.

7. Collection flask on the aspiration system must contain some bleach (i.e.,, enough to make solution 10% if full).

8. Always resuspend the cell pellet in the small quantity of liquid left after aspirating off the supernatant. Trying to resuspend cells in a larger volume of liquid, such as that added for washing, will result in a suspension of clumped cells. Recovery should be >50% (normally 90%) of retrievable cells. If the technologist is unfamiliar with lymphocyte isolation procedures, recovery should not be assessed until he or she is satisfactorily recovering >50% of the mononuclear cells. Inadequate recoveries are associated with failure to dilute the blood 1:2 with a buffer prior to layering over lymphocyte separation media, inadequate removal of cells from the plasma/lymphocyte separation media interface, failure to dilute the cells from the interface in Dulbecco's PBS, and inadequate centrifugation during the wash steps.

9. Viability of cells isolated by this method is usually 98%. Viability of cells should be assessed on all specimens by trypan blue exclusion.

10. Whole blood collected in late afternoon may be stored at room temperature and processed the next morning.

11. The level of CO_2 should be checked weekly with fyrite to determine % CO_2.

12. GLP and excellent sterile technique are very important because the cultures are maintained for up to 3 weeks and must remain free of contamination to give accurate results.

VIII. REFERENCES

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